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Identification of ciprofloxacin resistance by SimpleProbe™, High Resolution Melt and Pyrosequencing™ nucleic acid analysis in biothreat agents: *Bacillus anthracis*, *Yersinia pestis* and *Francisella tularensis*

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ABSTRACT

The potential for genetic modification of biological warfare agents makes rapid identification of antibiotic resistant strains critical for the implementation of suitable infection control measures. The fluorinated quinolone, ciprofloxacin, is an antibiotic effective for treating bacterial infections by inhibiting the enzyme activity of the DNA type II topoisomerases DNA gyrase and topoisomerase IV. The genes that encode the subunits of DNA gyrase (*gyrA* and *gyrB*) and topo IV (*par C* and *parE*) contain hotspots within an area known as the quinolone resistance-determining region (QRDR). Base pair changes within this region give rise to mutations that cause resistance to the antibiotic by altering amino acids within the enzymes. Ciprofloxacin-resistant (cipro^r) strains of *Bacillus anthracis*, *Yersinia pestis*, and *Francisella tularensis* with one or more known mutations within the QRDR of *gyrA*, *gyrB*, *parC*, and *parE* genes were tested with SimpleProbe™ and High Resolution Melt (HRM) dye chemistries and Pyrosequencing™ genetic analysis to evaluate the ability to rapidly detect ciprofloxacin-induced mutations. While SimpleProbe™ and Pyrosequencing™ successfully identified all known mutants, the HRM assay identified all but those resulting from G ↔ C or A ↔ T substitutions.

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1. Introduction

The anthrax attacks of 2001 demonstrated the use of a pathogenic organism as a bioweapon and highlighted the need for rapid and accurate diagnosis and response. In that event, 22 people became infected with *Bacillus anthracis* spores that resulted in five fatalities [1,2]. The ability to modify naturally occurring pathogens to become antibiotic resistant through genetic engineering further compounds the risk posed by potential biothreat agents. Knowledge of an organism's antibiotic resistance status is essential for timely and appropriate treatment decisions. The Department of Defense (DOD) has designated the fluorinated quinolone, ciprofloxacin, as one of the antibiotics of choice for treating *B. anthracis*, *Yersinia pestis*, and *Francisella tularensis* infections, as well as for post exposure prophylaxis [3]. Ciprofloxacin targets the bacterial type II enzymes, DNA gyrase (GyrA and GyrB) and topoisomerase IV (ParC and ParE), [4,5] and functions by stabilizing an intermediate stage of the DNA replication reaction thus inhibiting cell division [6,7]. Resistance to ciprofloxacin is caused by changes to amino acid sequences around the enzyme active site resulting in reduced drug affinity thereby allowing for continued bacterial cell growth. Genetic hotspots within the bacterial genome known as quinolone resistance-determining region (QRDR) of *gyrA*, *gyrB*, *parC* and *parE* genes give rise to mutations that cause resistance to ciprofloxacin (cipro^r) [8,9] by altering key amino acid residues in the topoisomerase II enzymes. Therefore, we have developed molecular assays that detect mutations within the QRDR regions of *B. anthracis*, *Y. pestis*, and *F. tularensis*. The mutants used in this study were generated from *B. anthracis* strain ΔANR, a plasmid cured Ames strain (pX01$^-$ & pX02$^-$) [10], *Y. pestis* KIM5, a pigmentation-negative (*pgm*$^-$) strain [11] and *F. tularensis* Schu4. For all assays, wt bacteria were exposed to increasing amounts of ciprofloxacin with selection of the resulting cipro^r mutants. For the SimpleProbe™ assays, primers were used to amplify segments within the QRDR genes flanking known mutation

Abbreviations: bp, base pair; QRDR, quinolone resistance determining region; wt, wild type; T_m, melting temperature; cipro^r, ciprofloxacin resistant; MIC, minimum inhibitory concentration.

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14. ABSTRACT
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sites. SimpleProbe™ probes, designed with sequences complementary to the wt, were used to determine the melting temperature (ΔT_m) difference between wt and mutants. Additional QRDR flanking primers were designed for use with High Resolution Melting (HRM) dye chemistry in which the resulting amplicons were differentiated by their ΔT_ms and identified by the change in melting curve shape [12]. Specific base pair analysis of the wt and mutant strains was also determined by Pyrosequencing™. This paper describes data resulting from these three methods for determining the cipro^r genetic status of *B. anthracis*, *F. tularensis*, and *Y. pestis*.

2. Materials and methods

2.1. DNA samples

2.1.1. B. anthracis

Wild-type *B. anthracis* ΔANR strain and ten cipro^r mutant clones were obtained from Dr. Lance B. Price [10]. Sequence data were provided for known mutations in the *gyrA*, *gyrB*, and *parC*, genes. All bacterial genomic DNAs were extracted and purified using the Qiagen (Valencia, CA) EZ1 according to the manufacturer's instructions under appropriate BSL conditions. The EZ1 is capable of up to 6 simultaneous extractions requiring approximately a total time of 45 min. In-house real-time PCR assays for plasmids pX01 (BAPA) and pX02 (BACAP) were performed to confirm the ΔANR phenotype in both the wt and mutants (data not shown).

2.1.2. Y. pestis

Wild-type *Y. pestis* KIM5 and five cipro^r mutant clones were obtained from Dr. Luther Lindler representing five different reported individual base changes within the *gyrA* gene [13].

2.1.3. F. tularensis

Cipro^r mutants were generated from *F. tularensis* strain Schu4 following standard microbiological techniques to develop antibiotic resistance. Colonies from an original chocolate agar culture were transferred to Mueller Hinton Broth supplemented with 2% Isovitalex (BBL, Cockeysville, MD) and containing 0.12 μg/ml ciprofloxacin. Wild-type Schu4 MIC is 0.25 μg/ml. Broth growth was serially passed through increasing concentrations of ciprofloxacin: 0.25, 0.5, 1, 2, 16 μg/ml. Incubations were at 35 °C for two weeks. Growth in broth containing 16 μg/ml ciprofloxacin was transferred to chocolate agar plates containing 64 μg/ml ciprofloxacin and incubated at 35 °C for 1 week. Colonies were picked, re-isolated and a standard MIC panel was run to determine ciprofloxacin susceptibility levels. Isolates were incubated in a BioLog GN plate (Biolog, Hayward CA) with a Schu4 control to ensure that the isolates were *F. tularensis*. Cipro^r isolates were sequenced with Big Dye™ (Applied Biosystems Inc., Foster city, CA) reagents following the manufacturer's instructions on an Applied Biosystems 3100 ABI PRISM automated DNA sequencer. The sequence data were analyzed by the programs contained in the Lasergene suit (DNASTAR, Inc., Madison, WI). Consensus sequences were determined by the SeqMan program (DNASTAR, Inc.). Alignment analysis of the consensus sequences was performed with the MegaAlign program (DNASTAR, Inc.) using the default settings of the Clustal W method (Table 1).

2.2. PCR assays

2.2.1. SimpleProbe™ assays

SimpleProbes™ were designed complementary to wt *B. anthracis* ΔANR, wt *Y. pestis* KIM5, and wt *F. tularensis* Schu4 spanning the regions within each gene where mutations were identified by sequence data [10,13] and data presented here. LightCycler (LC) Probe Design software V.2.0 (Roche Diagnostics, Indianapolis, IN) was used to design independent primers that amplified template DNA within the QRDR determining regions of each bacterial strain. Primer and probe sequences are listed in Table 2. Reaction conditions in 20 μL volumes were as follows: 3 mM $MgCl_2$ and 0.2 mM dNTP mix (Idaho Technology, Salt Lake City, UT); 0.2 μM SimpleProbes™ (Roche Diagnstics) with primer concentrations of 0.5 μM for the primer for the strand complimentary to the probe and 0.1 μM for the opposite strand, 1× concentration of exonuclease free KlenTaq LA polymerase combined with an equal volume of TaqStart antibody (Clontech Laboratories, Inc., Mountain View CA). Template DNA was added in a 5 μl volume for a final concentration of 100 pg/tube. Thermal cycling in LC 2.0 was performed as follows: one cycle at 95 °C for 2 min, followed by 50 cycles of 95 °C for 1 s, and 60 °C for 20 s with a fluorescence reading taken at the end of each 60 °C step. A melt curve was generated at the end of the amplification cycles by increasing the temperature to 95 °C at a programmed rate of 0.1 °C/s with a continuous read. The LC 4.0 software T_m calling and genotyping analysis modules were used to analyze the SimpleProbe™ melt curve data.

Table 1
Nucleotide sequences of the *F. tularensis* Schu4 mutations found in samples in the present study.[a]

Gene	Strain	Sequence (5′ → 3′)
gyrA	Wild-type	231 TCACCCCCATGGAGATACAGCTGTTTACGATA CTATTGTC
	F. tul Mut-127	231 TCACCCCCATGGAGATA**T**AGCTGTTTAC**T**AT ACTATTGTC
parE	Wild-type	640 ACATGGCATTTTGAAACTGGACTTAAAGGCT ATCTTGACC
	F. tul Mut-127	640 ACATGGCATTTTGAAACTGGAC*****GGCTAT CTTGACC

*Designates a 5-bp deletion.
[a] The mutation types are in bold face and underlined.

2.2.2. HRM assays

Oligonucleotide primers flanking each mutation site in the HRM assays were designed using Primer Express Version 2.0 for Windows (Applied Biosystems). The HPLC purified-primer sequences used in HRM PCRs are listed in Table 3. The 20 μl reaction mixture was Roche LightCycler 480 HRM dye kit at a 1× concentration, 3 mM $MgCl_2$ and 0.4 μM of each primer. Final concentration of DNA template was 100 pg/well. Reaction conditions in the LC 480 system were as follows: 10 min denature at 95 °C; 40 amplification cycles of 95 °C for 10 s, 60 °C for 10 s, 72 °C for 10 s, followed by a single read at 72 °C. After amplification the samples were heated to 95 °C for 1 min, cooled to 40 °C for 1 min and heated to 95 °C at a rate of 1 °C/s with 25 acquisitions/°C. Data were analyzed by the LC 480 Gene Scanning Software Module.

2.3. Pyrosequencing™

PSQ Assay Design software (Biotage AB, Uppsala, Sweden) was used to design the Pyrosequencing™ assays for the specific cipro^r mutations found in the QRDR regions of *gyrA*, *gyrB*, *parC* or *parE* of the *Y. pestis* KIM5, *B. anthracis* ΔANR and *F. tularensis* Schu4 clones. The software designed three oligonucleotides for each assay: two PCR primers (one of which was 3′-labeled with biotin) and a sequencing primer (Invitrogen, Carlsbad, CA) (Table 4). One hundred pg of genomic DNA of each clone was first amplified in a master mix containing 0.5 μM forward and reverse primer, 0.4 μL Platinum Taq (Invitrogen), Idaho Technology, Inc. (ITI) (Salt Lake City, UT) buffer with 5 mM $MgCl_2$, 2 mM dNTP, and 5 μL DNA in a total volume of 50 μL. An MJ Mini Thermocycler (Biorad, Hercules, CA) was used with the cycling conditions of: 95 °C for 2 min; 95 °C for 5 s and 60 °C for 20 s (40 cycles); and 72 °C for 5 min. The resulting

Table 2
SimpleProbe™ primer and probe sequences.

Organism	Target gene	Amplicon size (bp)	Primers and probes	Sequence (5′ → 3′)	Final conc (μM)
Bacillus anthracis	*gyrA*	88	F228	CGGTAAGTATCACCCTC	0.5
			R315	AACAAGCATATAACGTTGAC	0.1
			p274A	CCATCGTTTCATAAACAGCTGAAT-SPC[a]-Fluorescein	0.2
	gyrB-1	292	F1216	GTTTCAAGTTTACCTGGTAAATTAGC	0.5
			R1507	CCGTCATAATAATAACTTATGATAACGAG	0.1
			p1303A	CGGCAGAGTCACCCTCTACAA-SPC-Fluorescein	0.2
	gyrB-2	152	F1301	CCGGTGGATCAGCAAAG	0.1
			R1452	AATGTTCGTACCAATTGCAG	0.5
			p1397S	AAATCTTATCTAACGATGAAGTGCGTACAAT-SPC-Fluorescein	0.2
	parC	158	F179–2	CGTTCCGTAAGTCGGCTA	0.1
			R336	CCCGTCAACACTACCATTAT	0.5
			p235S	GGTGATTCCTCTGTATATGAAGCGATGG-SPC-Fluorescein	0.2
Francisella tularensis	*gyrA*	201	F98	CTTTGCCAGATGTGCGTGAT	0.1
			R298	AGCGCAATGAGAAAGGTTGTG	0.5
			p237S	TCATGGAGATACAGCTGTTTACGATA-SPC-Fluorescein	0.2
	parE	105	F619	AAAGAGAAGCTTACATGGCATTTTG	0.1
			R723	GAAATTATCGATCATAAACGGTTCTG	0.5
			p645S	AACTGGACTTAAAGGCTATCTTGACC-SPC-Fluorescein	0.2
Yersinia pestis	*gyrA*	185	F163	CTGGGTAATGACTGGAATAAACCA	0.5
			R347	GAGTCACCATCGACGGAACC	0.1
			p264A	Fluorescein-SPC-AGTGTCGTAGACCGCGCTGTCACCATGC-Blocker[b]	0.2

[a] SPC: SimpleProbe chemistry.
[b] 3′-end blocker.

amplicons were then run on the PyroMark ID Pyrosequencing™ instrument (Qiagen) according to the manufacturer's instructions. Sequence information was analyzed as to the presence or absence of the previously identified mutations.

3. Results

3.1. SimpleProbes and HRM

Fig. 1A and B are tracings of SimpleProbe™ and HRM assays for the *B. anthracis gyrA* results. These were selected because more samples were available with mutations to this gene and exhibited the largest variety of mutations. These figures are representative of the results obtained for the other gene assays. Table 5 summarizes data obtained for each organism where a mutation was found showing individual bp changes, T_ms of wt and mutants for the SimpleProbe™ assay and software calls for the HRM assays. The following sections describe specific SimpleProbe™ and HRM results for each bacterium.

3.1.1. B. anthracis

Wt and cipro^r mutants of *B. anthracis* ΔANR strain were amplified by primers flanking an 88 bp segment for *gyrA*, 292 bp for *gyrB*-1, 152 bp for *gyrB*-2 and 158-bp for *parC* of the QRDR coding region. Ten *B. anthracis* ΔANR isolates containing three different *gyrA*, two *gyrB*, and two *parC* point mutations were identified as mutants by SimpleProbe™ and HRM assays (Table 5). An example of the SimpleProbe™ melting profiles for the *B. anthracis gyrA* wt and mutants is shown in Fig. 1A. The HRM normalized melting curves for *B. anthracis gyrA* are shown in Fig. 1B. All *B. anthracis* clones, including S2–3, (G253 → A) were identified as wt by a SimpleProbe™ extending from position 249 to 280 of the *parC* gene (data not shown). However, clone S2–3 was identified as a mutant by a SimpleProbe™ spanning position 235 to 262 of the *parC* gene as demonstrated by a $T_m \sim 5\ ^\circ C$ less than that of the wt. Clone S2–3 was also identified as a mutant by the HRM assay. However clone S3–16 with two mutations was called wt by the HRM assay due to the C254 → T and A266 → C mutations which balanced each other thermodynamically and as a result did not significantly affect the overall T_m of the resulting amplicon.

Table 3
HRM primer sequences.

Organism	Target gene	Amplicon size (bp)	Primers	Primer sequence (5′ → 3′)
Bacillus anthracis	gyrase A	58	F229	GGTAAGTATCACCCTCATGGTGATT
			R286	GCGCCATACGTACCATCGTT
	gyrase B-1	72	F1242	AGATTGCTCTTCAAAAGATCCAGC
			R1313	GCTGATCCACCGGCAGAGT
	gyrase B-2	85	F1351	CCACTGAAAGGTAAAATTATTAACGTTG
			R1435	CAGTAATAATTGTACGCACTTCATCGT
	par C	64	F222	CTATCACCCGCACGGTGATT
			R285	CGTACTTTCCAAGTTTGACTTAAACG
Francisella tularensis	gyrase A	65	F221	TCGGTAAATATCACCCTCATGGAG
			R285	AGGTTGTGCCATTCTGACAATAGTAT
	par E	58[a]	F628	CTTACATGGCATTTTGAAACTGGAC
		53[b]	R685	CAGCTTCTAGTTTATGGTCAAGATAGCC
Yersinia pestis	gyrase A	53	F220	ATCGGTAAATACCACCCGCAT
			R272	CGCACGATAGTGTCGTAGACCG

[*] *F. tularensis* had a 5 bp deletion.

Table 4
Pyrosequencing assay primers.

Organism	Sequence (5′ → 3′)	Mutation detected (bp)
B. anthracis ΔANR		
gryA:		
F196[a]	AAA AAA TCA GCA CGT ATT GTT GG	
R334-BIO[b, c]	CAA AGT TAC CAT GCC CAT CA	
S223[d]	GTA ATC GGT AAG TAT CAC CCT CAT G	254C → T; G265 → A; A266 → C
gyrB:		
F1163-BIO	GTG TTG CAG CGA AAA AAG C	
R1469	ATA TCA AAA TCT CCG CCA ATG T	
S1309	5′-ATC CAC CGG CAG AGT-3′	G1309 → A
S1431	AAT AAT TGT ACG CAC TTC AT	A1423 → G
parC:		
F177	AGC GTT CCG TAA GTC GGC TAA A	
R342-BIO	CGG ATC CCC GTC AAC ACT	
S227	ACC CGC ACG GTG ATT	C242 → T[e]
Y. pestis KIM5		
gryA:		
F189	CAA AAA ATC GGC CCG TGT AG	
R341-BIO	CCA TCG ACG GAA CCG AAG TTA C	G241 → T; G242 → A; G248 → T;
S226	AAA TAC CAC CCG CAT	C249 → A; C249 → G
F. tularensis Schu4		
gryA:		
F222	CGG TAA ATA TCA CCC TCA TGG	
R318-BIO	TTG TCC ATC TAC TAG CGT ATA GCG	
S231	TCA CCC TCA TGG AGA	C248 → T; G259 → T
parE:		
F625	AAG CTT ACA TGG CAT TTT GAA AC	
R694-BIO	GGA GTG TTT CAG CTT CTA GTT TAT GGT	
S625	AAG CTT ACA TGG CAT TTT GAA AC	del: bp 653–657 (TTAAA)

[a] F – Forward (Upstream) primer.
[b] R – Reverse (downstream) primer.
[c] Biotinylated.
[d] S – Sequencing primer.
[e] Pyrosequencing was unable to detect the G253 → A as previously reported [6].

3.1.2. *Y. pestis*

Wt and cipro[r] mutants of *Y. pestis* KIM5 strain were amplified with primers flanking a 184 bp segment of the *gyrA* QRDR region. Single base changes are detected by a SimpleProbe™ designed with sequences complementary to *Y. pestis* KIM5 wt. HRM primers amplified a 53 bp segment before melting analysis. Because no reports were found regarding *parE*, the *parE* genes of the cipro[r] clones were sequenced with no mutations found (data not shown). Also reported were two KIM5 mutants with a bp change at C249 [14]. One of these, mutant M4, was correctly identified with both SimpleProbe™ and HRM. However, mutant M5 (C249 → G) was only detected by the SimpleProbe™ assay, as the bp change did not have a sufficient ΔT_m to be identified by HRM (Table 5).

3.1.3. *F. tularensis*

Sequencing of the *gyrA*, *gyrB*, *parC*, and *parE* genes of the single *F. tularensis* mutant clone was found to have one previously reported mutation, C248 → T, [15] as well as a new second G259 → T, in the *gyrA* gene. This mutant also had an unexpected five-bp deletion in the *parE* gene (Table 1). The SimpleProbe and HRM assays easily differentiated the wt from the mutant in assays for both the *gyrA* and the *parE* genes. The deletion of TTTAA in the *parE* gene resulted in an increase in the overall percentage of the GC content and thus, as expected, a higher T_m in the HRM assay as compared to that of the wt.

3.2. *Pyrosequencing*™

The PCR primers for Pyrosequencing™ detection of all known *gyr*A, *gyr*B, *par*C and *par*E cipro[r] mutations in *B. anthracis*, *Y. pestis* and *F. tularensis* are listed in Table 4. Each PCR primer pair was specifically designed to flank all nucleotide changes so that the third primer (sequencing) was able to detect the mutations during the Pyrosequencing™ reaction. The PCR reactions resulted in a range from a small 70-bp amplicon (*par*E of *F. tularensis*) to a large 307-bp amplicon (*gyr*B of *B. anthracis*). The 307-bp amplicon was necessary because the two mutations are 114 bp apart. Therefore, two unique sequencing primers were also needed to detect the single bp mutations with the *B. anthracis gyr*B amplicon. In all cases, Pyrosequencing™ was able to identify each specific nucleotide change within the topoisomerase QRDR regions of all but one cipro[r] mutant. The G253 → A mutation, as previously reported for *B. anthracis* ΔANR clone 2–3 [10], was not detected by either

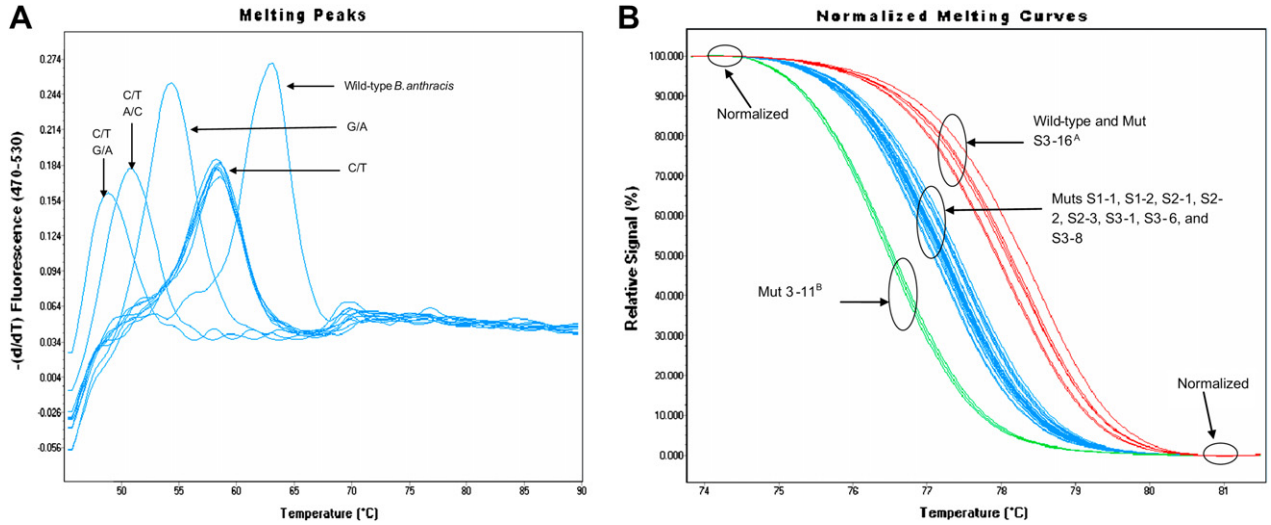

Fig. 1. A. SimpleProbe melting profile of ΔANR wild-type and mutants for the *B. anthracis gyr*A gene. B. HRM normalized melting curves of *B. anthracis gyr*A gene. [A]Mutant S3–16 had C → T and A → C mutations. [B]Mutant 3–11 had C → T and G → A mutations.

Table 5
Summary of mutations, SimpleProbe™ T_ms and HRM Gene Scanning module analysis of wild-type and cipro^r mutants.

A. *Y. pestis*	gyrA		
Strain	Δ Base	SP (T_m °C)	HRM
KIM 5 wt		76.27	wt
M1	G242 → A	71.95	mut
M2	G241 → T	72.58	mut
M3	G 248 → T	69.86	mut
M4	C249 → A	71.97	mut
M5	C 249 → G	72.12	wt

B. *F. tularensis*	gyrA			*parE*		
Strain	Δ Base	SP (T_m °C)	HRM	Deletion	SP T_m °C	HRM
Schu4 wt		59.87	wt		67.50	wt
Ft-127[a]	G248 → T G259 → T	50.06	mut	653 → 657 (TTTAA-del)	57.18	mut

C. *B. anthracis*	gyrA			gyrB(1)[c]			gyrB(2)[c]			parC		
Strain	Δ Base	SP (T_m °C)	HRM	Δ Base	SP (T_m °C)	HRM	Δ Base	SP (T_m °C)	HRM	Δ Base	SP (T_m °C)	HRM
ΔANR wt		62.56	wt		66.12	wt		66.62	wt		67.18	wt
S1–1	C254 → T	57.96	mut		66.21	wt		66.57	wt		67.15	wt
S1–2	G265 → A	53.99	mut		66.35	wt		66.64	wt		66.95	wt
S2–1	C254 → T	57.92	mut		66.23	wt		66.56	wt	C242 → T	61.39	mut
S2–2	C254 → T	57.92	mut		66.17	wt		66.63	wt	C242 → A	60.90	mut
S2–3	C254 → T	57.92	mut		66.44	wt		66.58	wt	C242 → A[d]	61.92	mut
S3–1	C254 → T	57.86	mut		66.11	wt		66.52	wt	C242 → T	61.47	mut
S3–6	C254 → T	57.76	mut		66.17	wt	A1423 → G	62.21	mut	C242 → T	61.63	mut
S3–8	C254 → T	57.81	mut	G1309 → A	57.16	mut		66.46	wt	C242 → T	61.48	mut
S3–11[b]	C254 → T G265 → A	48.98	mut		66.21	wt		66.37	wt	C242 → T	61.52	mut
S3–16[b]	C254 → T A266 → C	50.57	wt		66.25	wt		66.46	wt	C242 → T	61.77	mut

[a] *F. tularensis* mutant Ft-127 had two mutations in the *gyrA* gene and a five bp deletion in the *parE* gene.
[b] *B. anthracis* mutants S3–11 and S3–16 had two mutations in the *gyrA* gene.
[c] The distance between mutations in the *B. anthracis gyrB* gene required design of two probes.
[d] A G253 → A mutation was reported but a C242 → A mutation was found in this study.

SimpleProbe™ or HRM assays and its absence was verified by Pyrosequencing™. In addition, Pyrosequencing™ was able to locate the 5-bp deletion found in cipro^r *F. tularensis par*E gene of our newly isolated cipro^r clone.

4. Discussion

The purpose of this study was to evaluate rapid molecular assays to identify genetic mutations that confer resistance to ciprofloxacin in *B. anthracis*, *Y. pestis* and *F. tularensis*. We used the mutations previously published for *B. anthracis* and *Y. pestis* and our results for *F. tularensis* to design molecular assays for each gene where bp changes were found. The first gene affected at the lowest concentration of ciprofloxacin in each of the mutants of all three organisms was the *gyrA* gene [10,13] (data not shown). The role of bacterial GyrA enzyme in antibiotic sensitivity as well as the *gyrA* gene as loci for mutations that cause resistance is well documented. Type II topoisomerase DNA GyrA is a primary target of quinolones causing an irreversible DNA–gyrase complex resulting in cell death [6,16]. It has also been determined that quinolone resistance in *Escherichia coli* results from a mutation in the *gyrA* gene by causing a single amino acid change at the native Ser 83 [17]. Yoshida et al., [18] demonstrated that two independently isolated *E. coli* clones resistant to the quinolone nalidixic acid with amino acid changes at Ser 83 in the GyrA enzyme had sensitivity to quinolones restored by replacement of a DNA fragment containing the same segment of the *gyrA* wt gene. They also found that mutations to the *gyrA* gene that conferred resistance were located within a small region of the GyrA subunit (amino acids 67–106) known as the QRDR. The crystal structure of the GyrA topoisomerase enzyme reveals the identity and positions of amino acids in the QRDR that cluster around the tyrosine active site of the DNA–gyrase complex [19,20]. In studies reviewed by Hooper [21] and by Piddock [22] amino acid substitutions in both Gram positive and Gram negative bacteria also occur in the GyrA subunit in equivalent positions at Ser 83 and Asp 87. At least one cipro^r *gyrA* genetic mutation was found in each clone of all three organisms, *B. anthracis*, *Y. pestis* and *F. tularensis*, in locations relative to those reported in other cipro^r bacteria. Lindler et al., [13] found substitutions to Gly 81 and Ser 83 in *Y. pestis* while our newly isolated *F. tularensis* cipro^r mutant had amino acid substitutions at Thr 83 and Asp 87. Although the *parC* gene is reported to be the primary target of quinolones in other Gram-positive bacteria [23], Price et al., [10] found genetic mutations occurring within the *gyrA* gene of *B. anthracis* to be the most frequent resulting mostly in changes to Ser 85. The prevalence of mutations in this localized area in the *B. anthracis*, *Y. pestis* and *F. tularensis gyrA* gene indicate that it is a logical target for screening ciprofloxacin-resistance following species identification. It was previously determined that there were no mutations in the *parE* gene of cipro^r *B. anthracis* [10] and the finding of a *parC* mutation in *B. anthracis* is in keeping with the occurrence in other Gram-positive bacteria [24,25]. Subsequent testing and detection of mutations in the *gyrB*, *parC* and *parE* genes would indicate a potential increase in antibiotic sensitivity and MIC. While there may be mutations elsewhere in the *gyrA* gene, it is DNA bp changes that result in amino acid substitutions around this critical region near the enzyme active site known as QRDR hotspots that alter the enzyme–drug complex and are responsible for reduced drug affinity. This manuscript reports the detection of resistance to ciprofloxacin resulting from specific chromosomal genetic mutations. The authors acknowledge the possibility of other mechanisms

of resistance that protect bacteria from antibiotics. Price et al. [10] noted evidence of CIP resistance at low levels of the antibiotic caused by a chromosomal encoded efflux mechanism. However, plasmid mediated immunity to ciprofloxacin has not been demonstrated to be present in the bio-threat agents addressed here and further research into this possibility would provide valuable insight into fluoroquinolone resistance in these important pathogens [26].

Because the accumulation of specific single-base pair mutations within the QRDR region result in varying levels of ciprofloxacin resistance, we decided to develop diagnostic assays utilizing three distinct mutation-detection molecular technologies: SimpleProbes™, High Resolution Melt and Pyrosequencing™. The data shown in Table 5 reflect difference between SimpleProbe™ and HRM technologies. SimpleProbes™ detect mutations only between specific complementary nucleotide sequences within an amplicon with any mismatch destabilizing the probe/template complex thus altering the T_m. The nonspecific HRM dye relies on the melting profile of the entire amplicon and is characteristic of its GC content in which a substitution of a G or C to an A or T reduces the T_m while a substitution of an A or T to a G or C increases the T_m. The difference in T_ms of the wt and mutants obtained with the SimpleProbe™ is significant and easily distinguished and is reported as °C. Although HRM primers were designed so amplicon lengths were small to enhance sensitivity to single bp changes, T_ms obtained by melting curve analysis were routinely less than 1 °C. HRM melting curves were first normalized as described previously [27] before calculation by the LC 480 HRM software and thus reported as wt or mutant. Pyrosequencing™ using appropriately-designed PCR amplification and sequencing oligonucleotides, straightforwardly determined the exact nucleotide sequence within the QRDR regions of each cipro^r clone. The evaluation of QRDR mutations by Pyrosequencing™ can conclusively determine the ciprofloxacin sensitivity status of any test isolate.

The three assay technologies used in this study have both advantages and disadvantages. Our SimpleProbes™ are oligonucleotides designed to be 100% complementary to a particular wt gene sequence and should be capable of detecting all known ciprofloxacin-induced mutations within the QRDR regions of any *B. anthracis*, *Y. pestis* or *F. tularensis* isolate (pathogenic or non-pathogenic) because the assay primers were designed to the non-mutated sequences surrounding these regions. The advantage of this method is that annealing to mismatched sequences of mutant DNA and subsequent melting analysis, results in clearly lower T_ms than that of the wt. However, SimpleProbe™ design constraints limit their reactivity to a relatively short sequence within an amplicon. In contrast, the LC 480 HRM dye is a nonspecific double-stranded DNA saturating dye that enables detection of sequence variations without the need for a sequence specific probe based assay [28]. HRM data utilized the LC Gene Scanning Software Module to detect changes in normalized melting curve shapes due to minute differences in T_m caused by as little as a single nucleotide mutation. While HRM assays are less costly, C ↔ G or A ↔ T mutations are not likely be detected (see *Y. pestis* sample M5). In addition, even though *B. anthracis* S3–16 had double mutations (C → T and A → C) and a $T_m > 10$ °C of the wt in the SimpleProbe™ assay, the mutations thermodynamically cancelled each other out in the HRM assay. Pyrosequencing™ not only detects mutations, but pinpoints the specific bp change from the wt sequence. It is, however, a longer assay requiring two separate instruments with sequencing following a standard PCR amplification step. In contrast, SimpleProbe™ and HRM are closed system assays where real-time PCR amplification and melting profiles are performed in a single instrument.

While our SimpleProbe™ and HRM assays were done on a LightCycler 480 with results analyzed by the companion Roche Tm and Gene Scanning software, Herrmann, et al., [27] concluded that only the specific dye, LCGreen, was useful for HRM heterozygote amplicon scanning. Therefore, only instruments compatible with LCGreen (argon laser were specifically eliminated because of spectral mismatch) and specifically designed for HRM should be used due to their higher melt temperature scanning sensitivity and specificity capabilities. In our hands, SimpleProbe™ and HRM assays require approximately 1 hour for an instrument run and the follow-on data analysis on the LightCycler 480. Other SimpleProbe™ and HRM-capable instruments vary in their run and data analysis times.

In conclusion, bacterial resistance to antimicrobial drugs by spontaneous mutations is currently a serious public health concern and the danger posed by modification of biological agents could be catastrophic in the event of a deliberate release of one of these pathogens. Because rapid identification of antibiotic resistant organisms is such an important factor in determining appropriate and expeditious therapy, the three PCR technologies described in this study were evaluated for the ability to accurately differentiate wt *B. anthracis*, *Y. pestis* and *F. tularensis gyrA* from antibiotic resistant mutants following pathogen identification. The results presented here show rapid molecular assays that target mutations in a highly conserved region of the *gyrA* gene, thereby affecting the function of topoisomerase enzymes, can be used to identify potential cipro^r biothreat agents.

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